added by this amendment. Reconsideration is respectfully requested.

## I. Rejection of Claims 16 and 17 under 35 U.S.C. §101

Claims 16 and 17 have been rejected under 35 U.S.C. §101 as being directed to non-statutory subject matter. Specifically, the Examiner suggests that the claims recite an antibody that recognizes an extracellular portion of a BCRP, wherein said extracellular portion of the BCRP is in its natural conformation. Because the recited antibody may be present naturally, such as in the serum of a leukemia patient, the claims are suggested to read on claims of nature. As suggested by the Examiner, and in an effort to facilitate prosecution and clarify the invention, claims 16 and 17 have been amended to recite an "isolated" antibody. Support for this amendment is found throughout the specification and especially at page 22, lines 29-31. To further define the isolated antibody of the invention, new claims 21-24 were added, as supported throughout the specification and especially at page 9 in lines 21 through 29.

Withdrawal of this rejection is therefore respectfully requested.

**II. Objections to Claim 17**

The Examiner has objected to Claim 17 as being of improper dependent form for failing to further limit the subject matter of a previous claim. In an earnest effort to facilitate prosecution, claim 17 has been amended to present the claim in independent format. Support for this amendment is found throughout the specification and at claims 16 and 17 as filed. Applicants believe that this amendment overcomes the objection to this claim. Therefore, Applicants respectfully request withdrawal of the objection to the claim.

**III. Rejection of Claim 16 and 17 under 35 U.S.C. §102(a)**

Claims 16 and 17 are rejected under 35 U.S.C. §102(a) as being anticipated by Scheffer et al.(*Proc. Am. Assoc. Cancer Res.* 2000 Mar; 41:page 803). The Examiner suggests that Scheffer et al. teach a monoclonal antibody for detection of a BCRP protein in the cell membrane (surface) of the cells from MCF-7 sublines, i.e., an antibody recognizing the extracellular portion of the BCRP protein on the cell surface. The Examiner further suggests that a BCRP would inherently be present on the surface of a viable stem cell, and therefore the Examiner suggests that

Scheffer et al., anticipate the present invention. Applicants respectfully disagree.

To anticipate a claim, a reference must teach every element of the claim. See MPEP § 2131. The cited reference does not teach every element of the claimed invention.

The Scheffer et al. abstract suggests that BXP-34 monoclonal antibody could potentially recognize an extracellular portion of the BCRP protein. However, in the paragraph bridging pages 2591 and 2592 of the enclosed Scheffer reference (Scheffer et al., *Cancer Research*, 60 2589-2593, May 15, 2001), the authors further detail the characteristics of the BXP-34 monoclonal antibody and teach that the antibody detects an **internal** epitope of the BCRP protein. This reference is conclusive evidence that the antibody does not recognize any extracellular portion of BCRP and therefore cannot be held to anticipate the present invention.

Withdrawal of this rejection is respectfully requested.

## IV. Rejection of Claims 16 and 17 under 35 U.S.C. §103

Claims 16 and 17 are rejected under 35 U.S.C. §103(a) as being unpatentable over Ross et al. (hereinafter Ross) in view of Niman et al.(hereinafter Niman). The Examiner suggests that Ross

teach the BCRP protein is a novel xenobiotic transporter which is overexpressed in a variety of human cancer cells. Ross is suggested to recite an objective of providing antibodies to BCRP, at column 2, line 30 of the reference. The Examiner suggests that since only the extracellular portion of the protein on the cell surface is accessible to antibody recognition in living cells, the antibody taught by Ross is suggested to meet the claim limitations, i.e. recognizing the extracellular portion of the BCRP protein on the cell surface, and BCRP is suggested to inherently be present on the surface of a viable stem cell. The Examiner acknowledges that Ross does not actually disclose an anti-BCRP antibody in the specification. The Examiner suggests, however, that at the time of filing, it was well known in the art how to make an antibody from a protein or polypeptide. The Examiner suggests that the abstract and claims 1-9 of the Niman reference teach the method of making an antibody against the cell receptor polypeptides. Therefore, the Examiner reasons that it would have been obvious to one of ordinary skill in the art at the time of the invention to employ the methods taught by Niman to make an antibody to BCRP as taught by Ross with a reasonable expectation of success. Applicants respectfully disagree.

To establish a *prima facie* case of obviousness under 35 U.S.C. 103(a) three basic criteria must be met. MPEP § 2143. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art must teach or suggest all of the claim limitations.

The cited references fail to meet all of these criteria with respect to the instant claimed invention.

Ross teaches the preparation of antibodies using purified BCRP at column 4, lines 50-57. Since antibodies generated against purified protein may recognize any portion of the protein, the suggestion that the antibody taught by Ross meets the claim limitations of claims 16 and 17 is incorrect. There is no teaching in Ross for generating antibodies which will specifically recognize the extracellular portion of BCRP, nor is there any teaching or suggestion of isolating such antibodies from a generic preparation of antibodies made against purified BCRP protein. Further, the antibodies of Ross are prepared against a purified protein. A purified protein is different than the natural conformation required by Applicants' claims.

Accordingly, there is no teaching that the Ross antibodies recognize the protein in its natural conformation.

Further, Niman does not provide any additional teaching or suggestion sufficient to remedy the deficiencies of Ross. Niman fails to teach any method for generating an antibody that recognizes an extracellular portion of a protein in its natural conformation. The generation of an antibody to a small peptide representing a portion of BCRP as taught by Niman is not the same as an antibody which will recognize the corresponding portion of BCRP in its natural conformation as required by the claims of the present invention. Accordingly, the combination of Ross and Niman fail to provide the information necessary to teach or suggest all of the claim limitations of the present invention.

Withdrawal of this rejection is respectfully requested.

## V. Conclusion

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "Version with Markings to Show Changes Made."

Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly,

favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

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Date: **March 4, 2003**

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**Version with Markings to Show Changes Made**

**In the Claims**:

Claims 1-15 and 18-20 have been canceled.

Claims 16 and 17 have been amended as follows:

16. (Amended) An isolated antibody that recognizes an extracellular portion of a BCRP; wherein said extracellular portion of the BCRP is in its natural conformation.

17. (Amended) ~~The antibody of Claim 16~~ An isolated antibody that recognizes an extracellular portion of a BCRP; wherein said extracellular portion of the BCRP is in its natural conformation, and wherein the BCRP is on the surface of a viable stem cell.

[CANCER RESEARCH 60, 2589–2593, May 15, 2000]



*Advances in Brief*

# Breast Cancer Resistance Protein Is Localized at the Plasma Membrane in Mitoxantrone- and Topotecan-resistant Cell Lines[1]

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## Abstract

**Tumor cells may display a multidrug resistant phenotype by overexpression of ATP-binding cassette transporters such as multidrug resistance (*MDR1*) P-glycoprotein, multidrug resistance protein 1 (*MRP1*), and breast cancer resistance protein (*BCRP*). The presence of BCRP has thus far been reported solely using mRNA data. In this study, we describe a BCRP-specific monoclonal antibody, BXP-34, obtained from mice, immunized with mitoxantrone-resistant, *BCRP* mRNA-positive MCF-7 MR human breast cancer cells. BCRP was detected in *BCRP*-transfected cells and in several mitoxantrone- and topotecan-selected tumor cell sublines. Pronounced staining of the cell membranes showed that the transporter is mainly present at the plasma membrane. In a panel of human tumors, including primary tumors as well as drug-treated breast cancer and acute myeloid leukemia samples, BCRP was low or undetectable. Extended studies will be required to analyze the possible contribution of BCRP to clinical multidrug resistance.**

## Introduction

Tumor cells can be intrinsically resistant to drugs or they can acquire resistance to structurally and functionally unrelated drugs on drug exposure. This phenomenon is known as MDR[3] (reviewed in Ref. 1). In human tumor cells, several transporter proteins can be involved in MDR. These proteins—MDR1 P-gp (ABCB1; reviewed in Ref. 2), MRP1 (ABCC1; reviewed in Ref. 3), MRP2 (ABCC2; Ref. 4), MRP3 (ABCC3; Ref. 5), and BCRP (ABCG2; Ref. 6)—all belong to the ABC transporter family (7). They act as efflux pumps, which result in decreased intracellular concentrations of cytotoxic drugs.

BCRP is a recently discovered half-transporter that probably acts as a homo- or heterodimer in transporting cytotoxic agents (6). The transporter molecule is capable of transporting several anticancer drugs but has thus far been found mainly in MX-resistant cell lines (8, 9).

To date, all studies on *BCRP* expression have reported on *BCRP* mRNA levels. Because no polyclonal or monoclonal antisera that would detect BCRP are yet available, studies at the protein level have not yet been described. Therefore, information regarding the presence and localization of BCRP in (tumor) cells is still lacking.

The MCF-7 MR breast cancer cell line is one of several MX-resistant cell lines described with a non-P-gp, non-MRP1 phenotype and elevated levels of *BCRP* mRNA (8). To characterize the resistance mechanism in cells with these characteristics, we set out to produce Mabs reactive to proteins elevated in this cell line, as compared with sensitive cells. Mice were immunized with MCF-7 MR cells and, using a cytospin-based screening method with MX-resistant and -sensitive cell lines, we isolated a Mab named BXP-34 that specifically reacts with the BCRP protein. To study the presence and subcellular localization of BCRP in human tumor cell lines and tumor samples, panels of parental and MX-, TPT-, and multidrug-resistant cell lines as well as primary and chemotherapy-treated breast cancer and AML samples were tested for BCRP using the BXP-34 Mab.

## Materials and Methods

**Cell lines.** All of the cell lines that we used have been described previously: (*a*) the drug-sensitive breast cancer cell line MCF-7, the Dox-selected subline MCF-7 Dox40, the MX-selected subline MCF-7 MR, and the mock- and *BCRP* (clone 8)-transfected sublines (6, 10); (*b*) the myeloma cell line 8226, the Dox-selected subline 8226 Dox40, and the MX-selected subline 8226 MR20 (11); (*c*) the ovarian carcinoma cell line Igrov1, the TPT-selected subline T8, the partial revertant of this cell line, the T8rev, and the MX-selected subline MX3 (9); (*d*) the ovarian carcinoma cell line A2780 and the Dox-selected subline 2780AD (12); (*e*) the non-small cell lung cancer cell line SW1573 and the Dox-selected sublines SW1573/2R120 and SW1573/2R160 (13); (*f*) the small cell lung cancer cell line GLC4, the Dox-selected GLC4/ADR subline, and the MX-selected subline GLC4 MIT (14); and (*g*) the leukemia cell line HL60 and the Dox-selected HL60/ADR subline (15). The ovarian carcinoma cell line 2008 and the *MRP1*-, *MRP2*-, and *MRP3*-transfected sublines were described in Ref. 5 and by Scheffer *et al.*[4] All of these cell lines are human cell lines. The mouse fibroblast cell line MEF3.8, the MX-selected subline M32, and the TPT-selected subline T6400 were described in Ref. 16. Monkey kidney CV-1 cells, transformed by an origin-defective mutant of SV40 that codes for wild-type T antigen, COS7 cells, were described in Ref. 17.

All of the cell lines were grown in Dulbecco's modified essential medium or RPMI (Life Technologies, Inc. Europe, Paisley, Scotland), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin, and streptomycin. Resistant cell lines were cultured in the presence of drugs until 3–10 days

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[3] The abbreviations used are: MDR, multidrug resistance; ABC transporter, ATP-binding cassette transporter; P-gp, P-glycoprotein; MRP, MDR protein; BCRP, breast cancer resistance protein; Mab, monoclonal antibody; MX, mitoxantrone; TPT, topotecan; Dox, doxorubicin; CyPhos, cyclophosphamide; AML, acute myeloid leukemia; HRP, horseradish peroxidase; AEC, aminoethylcarbazole.

[4] G. L. Scheffer, M. Kool, M. Heijn, M. de Haas, A. C. L. M. Pijnenburg, J. Wijnholds, A. van Helvoort, M. C. de Jong, J. H. Hooijberg, C. A. A. M. Mol, M. van der Linden, J. M. L. de Vree, P. van der Valk, R. P. J. Oude Elferink, P. Borst, and R. J. Scheper. Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5 and MDR3 P-glycoprotein with a panel of monoclonal antibodies, submitted for publication.

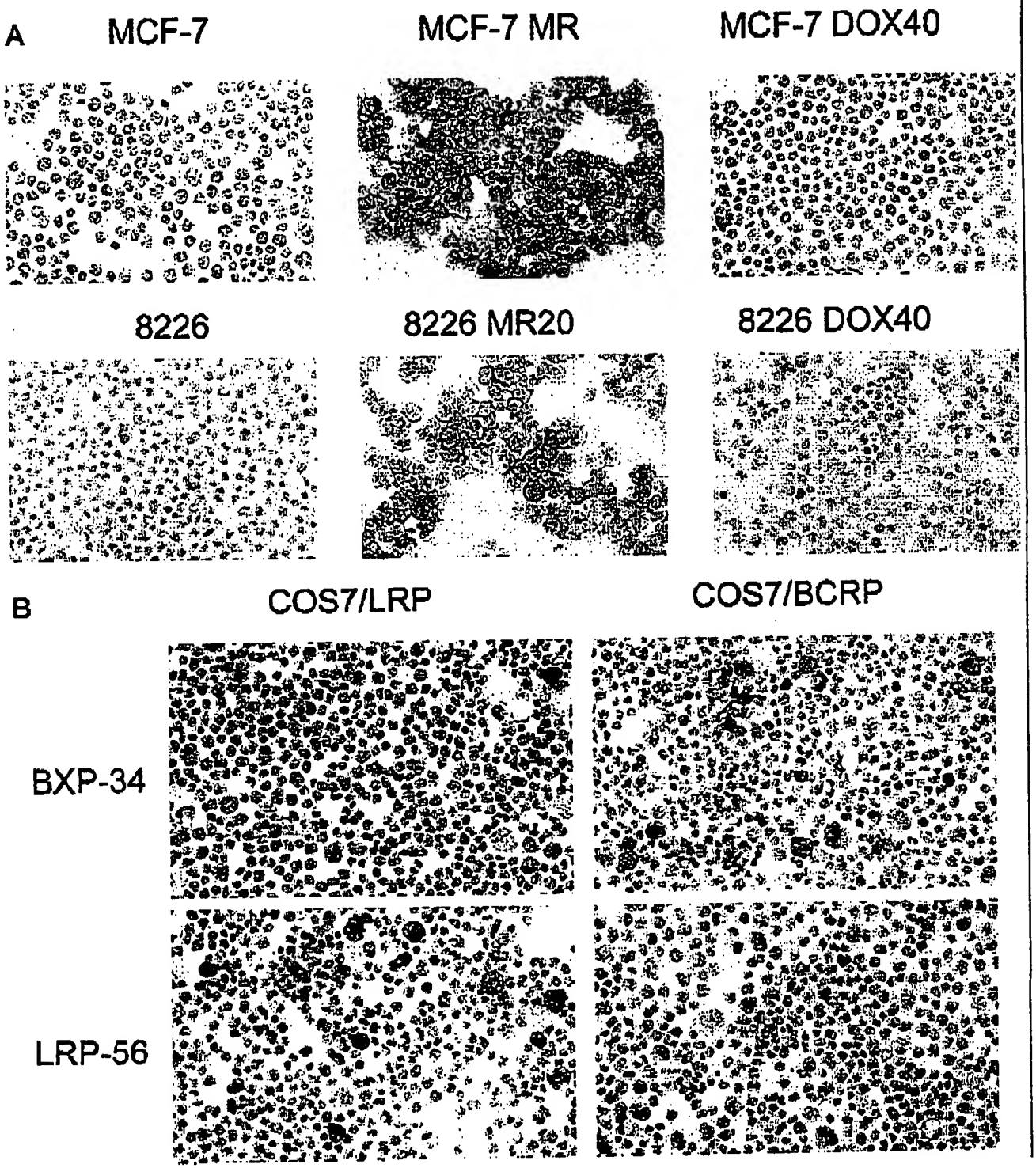


Fig. 1 *A*, staining of cytospins of parental and drug-selected sublines of MCF-7 (breast cancer) and 8226 (myeloma) human tumor cells with the BXP-34 Mab. The MR sublines are MX-selected and the Dox sublines are Dox-selected (see Table 1). *B*, COS7 cells transfected with *LRP* (control; *left panel*) or *BCRP* cDNA (*right panel*) stained with LRP-56 control Mab (*lower panel*) and BXP-34 Mab (*upper panel*). Biotinylated rabbit-antimouse serum and HRP-labeled streptavidin were used as secondary reagents. Color development was with AEC.

before the experiments. All of the cells were negative for *Mycoplasma* as tested by the Gene-Probe rapid *Mycoplasma* detection system (Gene-Probe, San Diego, CA).

**Immunizations and Mab Production.** Three 8–12-week-old female BALB/c mice (Harlan, Zeist, the Netherlands) received footpad injections of sonicated MCF-7 MR cells emulsified in Freund's complete adjuvant (Difco, Detroit, MI). Approximately $2 \times 10^6$ cells were used per injection. After 10 days, the animals received a first booster injection with sonicated MCF-7 MR cells in PBS. Similar booster injections were given at day 20 and 30. Four days after the last booster, 3 days before fusion, a final booster

Table 1 *Panel of human tumor cell lines stained with BXP-34 for BCRP*

Cell line, origin, drug used for cell line selection, MX resistance level, transporter protein present in the cell line, cell line reference, and BCRP staining result are indicated. In the panel of tumor cell lines, a good correlation between reported *BCRP* mRNA levels and staining with BXP-34 is observed.

| Cell line | Origin | Selective drug | RF[a] MX | Transporter | Reference | BXP-34 staining |
|---|---|---|---|---|---|---|
| MCF-7 | Breast cancer | No | 1 | (parent) | (10) | − |
| MCF-7 MR | | 80 nM MX | 1208 | BCRP +++[b] | | +++ |
| MCF-7 Dox40 | | 400 nM Dox | 153 | P-gp +++ | | − |
| MCF-7 mock | | No | 1 | (parent) | (6) | +/− |
| MCF-7/BCRP | | No | 32 | BCRP ++[b] | | ++ |
| 8226 | Myeloma | No | 1 | (parent) | (11) | − |
| 8226 MR20 | | 20 nM MX | 37 | BCRP +[b] | | ++ |
| 8226 Dox40 | | 400 nM Dox | 3.6 | P-gp ++ | | − |
| Igrov1 | Ovarian cancer | No | 1 | (parent) | (9) | − |
| Igrov1 T8 | | 950 nM TPT | 11 | BCRP ++[b] | | +++ |
| Igrov1 T8rev | | No | 4 | BCRP +/−[b] | | + (50%) |
| Igrov1 MX3 | | 340 nM MX | 11 | BCRP ++[b] | | +++ |
| SW1573 | Lung cancer | No | 1 | (parent) | (13) | − |
| SW1573/2R120 | | 120 nM Dox | ? | MRP1 ++ | | − |
| SW1573/2R160 | | 160 nM Dox | ? | P-gp +++ | | − |
| A2780 | Ovarian cancer | No | 1 | (parent) | (12) | − |
| 2780AD | | 2000 nM Dox | ? | P-gp +++ | | − |
| GLC4 | Lung cancer | No | 1 | (parent) | (14) | − |
| GLC4/ADR | | 1152 nM Dox | 27.5 | MRP1 +++ | | − |
| GLC4 MIT | | 60 nM MX | 60 | ? | | − |
| HL60 | Promyeloid | No | 1 | (parent) | (15) | − |
| HL60/ADR | | 350 nM Dox | ? | MRP1 ++ | | − |
| 2008 | Ovarian cancer | No | 1 | (parent) | (5) | − |
| 2008/M1 | | No | 6.9[c] | MRP1 +++ | | − |
| 2008/M2 | | No | 1.7[c] | MRP2 +++ | | − |
| 2008/M3 | | No | 1.7[c] | MRP3 +++ | | − |

[a] RF, resistance factor; ?, unknown; −, no reactivity; +/−, weak reactivity; +, very weak reactivity; ++, good reactivity; +++, very good reactivity.
[b] mRNA data.
[c] M. Kool, unpublished data.

injection was given. The mice were housed and treated in accordance with current regulations and standards of the Institutional Animal Ethics Committee.

The mice were killed and draining popliteal lymph nodes were removed and used for fusion with mouse myeloma Sp2/0 cells as described previously (5). Hybridoma supernatants containing monoclonal antibodies were screened on octo-spins containing eight cytospins of a mixture of MCF-7 MR and MCF-7 parental cells per slide. Antibody binding was detected as described in the "Immunohistochemistry" section. Hybrid cells that secreted antibodies of interest were selected and subcloned three times by limiting dilution. The isotype of the selected Mabs was determined using IsoStrips (Boehringer Mannheim).

**Immunohistochemistry.** Cytospin preparations and cryosections (4 μm) were air-dried overnight and fixed for 7 min in acetone at room temperature. The slides were incubated with undiluted hybridoma supernatant for 1 h at room temperature. Biotinylated rabbit-antimouse serum (1:150, Zymed, San Francisco, CA) and HRP-labeled streptavidin (1:500, Zymed), diluted in PBS/1% BSA, were used as secondary reagents. Color development was with 0.4 mg/ml AEC and 0.02% $H_2O_2$ as a chromogen.

**Transient Transfections.** Monkey kidney COS7 cells were transfected with *pcDNA3-BCRP* (6) or (control) *pCDM8-LRP* plasmids by the DEAE dextran (Promega Corporation, Leiden, the Netherlands) method as described by Aruffa and Seed (18). Three days after transfection, the cells were harvested, cytospin preparations were made, and transient gene expression was examined using BXP-34 and (control) LRP-56 Mabs.

**Tumor Samples.** A panel of human tumor samples comprising tumors of different origin and relative drug sensitivity was selected from our frozen tissue bank (see Table 1). For most tumor types, two patients were selected. The samples were mainly primary untreated adenocarcinoma samples. In the breast cancer group, more patients were included. Besides 10 patients with untreated adenocarcinoma, 7 patients with locally advanced breast cancer were entered. These latter patients had received standard combined Dox/CyPhos chemotherapy (six cycles with dosages starting at 90 mg/m² Dox and 1000 mg/m² CyPhos, decreasing to 75 mg/m² Dox and 750 mg/m² CyPhos in the last period).

Cytospins from paired samples from 8 patients with AML *de novo* and at relapse were made. Patients were treated with standard induction chemotherapy treatment that consisted of a first cycle of daunorubicin (45 mg/m²) or idarubicin (12 mg/m²) plus 1-β-D-arabinofuranosylcytosine (200 mg/m²) for 7 days, followed by a second cycle of amsacrine (120 mg/m²) plus 1-β-D-arabinofuranosylcytosine (2000 mg/m²) for 6 days and a third cycle of MX (10 mg/m²) plus etoposide (100 mg/m²) for 5 days (6 patients) or an autograft or HLA-matched allograft (2 patients).

## Results

**Mab Production.** To study the transporter(s) involved in MX-selected, non-P-gp non-MRP1 MDR cell lines, we set out to produce Mabs detecting proteins up-regulated in MX-resistant cell lines. Because *BCRP* mRNA levels were reported to be up-regulated in these cell lines, particular attention was given to this transporter. Hybridoma supernatants obtained from mice that had been immunized with *BCRP* mRNA-positive breast cancer MCF-7 MR cells were screened on cytospins for reactivity with MCF-7 MR cells and absence of reactivity with parental MCF-7 cells and P-gp-positive, *BCRP* mRNA-negative MCF-7 Dox40 cells. Several clones were selected and were further examined for reactivity with a similar panel of 8226 myeloma cell lines. This approach caused us to discard most of the clones because they failed to react with MX-resistant, *BCRP* mRNA-positive myeloma 8226 MR20 cells but resulted in the isolation of one Mab, called BXP-34, most likely to detect the BCRP protein (Fig. 1*A*).

**Characteristics of the BXP-34 Mab.** Specificity of reactivity of the BXP-34 Mab to BCRP was confirmed by reactivity with monkey kidney COS7 cells transiently transfected with *BCRP* cDNA (Fig. 1*B*). The Mab does not cross-react with known MDR transporters like P-gp, MRP1, MRP2, or MRP3, as shown by the absence of staining with cells overexpressing these transporter molecules (Table 1). The BXP-34 Mab was unreactive in Western blots with protein extracts of BCRP-positive cells (not shown), which indicates that the Mab reacts

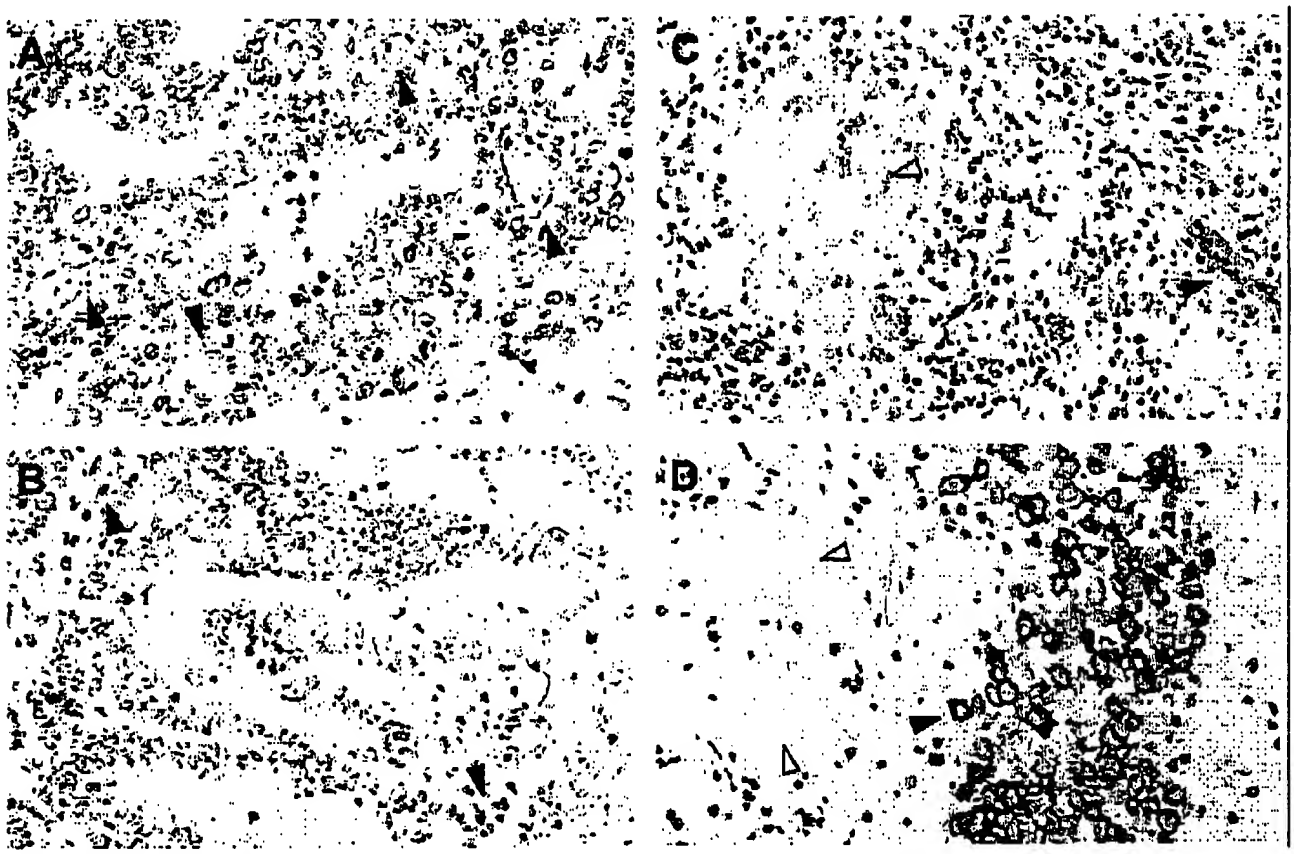


Fig. 2. Cryosections, stained with the BXP-34 Mab, of two small-intestine tumors (*A*, *B*); a testicular tumor (*C*); and a control section of normal human kidney, preinjected with a mixture of parental and MX-resistant MCF-7 MR breast cancer cells (*D*). Biotinylated rabbit-antimouse serum and HRP-labeled streptavidin were used as secondary reagents. Color development was with AEC. *A*, ▲, BCRP staining of the epithelial cells of the neoplastic crypts. *B*, no staining is observed, except for some desmoplastic stroma staining (▲). *C*, the seminoma cells are negative (△), but staining of the endothelial cells is observed (▲). *D*, no BCRP staining is observed in the kidney tubules (△), whereas strong staining is observed in the MCF-7 MR cells in the injected tumor cell suspension (▲).

with a nonlinear epitope of the protein. The Mab was unable to stain viable unfixed BCRP-positive cells, which showed that the Mab detects an internal epitope of the BCRP protein. The Mab was also unreactive on cytospins of *Bcrp* mRNA-positive mouse M32 and T6400 cells, which showed that the BXP-34 epitope is not preserved in mouse Bcrp. Isotyping of the Mab showed that BXP-34 is of IgG1 subclass.

**BCRP in Human Tumor Cell Lines and Tumor Samples.** Using the BXP-34 Mab on cytospins and frozen sections of a panel of human tumor cell lines and tumor samples, we examined the presence of BCRP (Figs. 1 and 2 and Tables 1 and 2). First, cytospins of a panel of parental cell lines, cell lines selected for resistance to TPT, MX, or Dox, and cell lines transfected with MDR transporters were examined. As shown in Table 1, the BXP-34 Mab detected no, or very low, amounts of BCRP in the parental cell lines or in resistant cell lines with high levels of P-gp or MRP1. In contrast, in the MX-selected MCF-7 MR, the 8226 MR20, and the MX3 cell lines as well as in the TPT-selected T8 cell line, elevated levels of BCRP were detected. Staining of the cell membranes of these cells is most pronounced, with some additional cytoplasmic staining. BCRP levels in the T8rev, a partial revertant of the T8 cell line, were markedly decreased, in accordance with decreased *BCRP* mRNA levels and reduced resistance to TPT and MX (9). No staining was observed in the MX-selected GLC4 MIT cells. Also, no BXP-34 staining was observed in the 2008 sublines transfected with *MRP1*, *MRP2*, or *MRP3*. In the *BCRP*-transfected MCF-7/BCRP (clone 8) cells, as expected, clear membranous staining of BCRP was observed.

Then, BCRP levels were examined in cryosections of a panel of primary and chemotherapy-treated human tumor samples. As shown in Table 2, BCRP proved to be undetectable in all of the samples examined, except for a case of small-intestinal adenocarcinoma, in

Table 2 *Panel of human tumor samples stained with BXP-34 for BCRP*

Tumor type, number of samples, drug treatment, and BXP-34 staining are indicated. Except for one case of small intestine cancer, no BCRP presence was observed in the tumor cells of these tumor samples. Occasional reactivity of endothelial cells and desmoplastic stroma was observed in some tumor samples.

| Tumor type | *n* | Drug treatment | BXP-34 staining |
|---|---|---|---|
| Stomach, AC[a] | 2 | No | –[b] |
| Lung, AC | 2 | No | – |
| Colon, AC | 2 | No | – |
| Small intestine, AC | 1 | No | + |
| Small intestine, AC | 1 | No | – |
| Testis, Sm | 2 | No | – |
| Ovarium, AC | 2 | No | – |
| Kidney, AC | 2 | No | – |
| Liver metastases, AC | 2 | No | – |
| Hepatocellular, AC | 2 | No | – |
| Prostate, AC | 2 | No | – |
| Bladder, TCC | 2 | No | – |
| Uterus, AC | 2 | No | – |
| Breast, AC | 10 | No | – |
| Breast, LABC | 7 | Dox/CyPhos | – |
| AML | 8 | No | – |
| AML | 2 | Several | – |
| AML | 6 | Several + MX | – |

[a] AC, adenocarcinoma; Sm, seminoma; TCC, transitional cell carcinoma; LABC, locally advanced breast cancer.
[b] –, no reactivity; +, weak reactivity.

which weak staining of the apical membrane of the epithelial cells of the neoplastic crypts was observed (Fig. 2*A*). In some tumor samples, occasional staining of endothelial cells and desmoplastic stroma and inflammatory cells was observed (Fig. 2, *B* and *C*). Clear positive staining of tumor cells was observed in control cryosections from normal human kidney, preinjected with a cell suspension of MCF-7 MR and parental cells (Fig. 2*D*). In breast tumor samples of patients treated with combined Dox/CyPhos chemotherapy and in cytospins of *de novo* and relapsed AML patients, no detectable levels of BCRP were observed.

## Discussion

To date, of at least two ABC proteins, MDR1 P-gp and MRP1, the contribution to MDR in human tumor cells is well established. Some family members, including the recently cloned BCRP protein, are also able to transport anticancer drugs (5, 6, 19, 20). Whether they also play a role in drug resistance in patients remains to be established. We have recently described a panel of transporter-specific Mabs, facilitating such investigations.[4] Extending our studies we set out to produce Mabs detecting the putative MX transporter, the BCRP protein. Using MX-resistant, *BCRP* mRNA-positive, MCF-7 MR cells for immunization and a cytospin-based screening system with MX-resistant and -sensitive cells, we selected one BCRP-specific Mab, BXP-34. Several other Mabs that were initially selected for reactivity to MCF-7 MR cells were not reactive to other MX-resistant cell lines. These results indicate that (*a*) human BCRP is only weakly immunogenic in mice; and (*b*) proteins other than BCRP are selectively up-regulated in MCF-7 MR cells. Notwithstanding, using the BXP-34 Mab, all of the MX- and TPT-selected human cell lines tested were clearly found to be BCRP-positive, except for the GLC4 MIT cell line. In these cells, other mechanisms of MX resistance should be operative. The absence of *BCRP* mRNA as well as changes other than BCRP overexpression were described in some MX-resistant cell lines (8, 11). As anticipated from drug efflux studies (6, 9, 11), we show here that BCRP is localized most prominently at the plasma membrane rather than at internal vesicular membranes. The BCRP transporter is, therefore, more likely to be involved in active transport from the cell than in transport into internal vesicles.

Except for a case of small-intestine cancer that was weakly positive, cryosections of a panel of human primary tumors were BCRP-negative. Even in tumors, such as renal adenocarcinoma, that have a relatively high level of intrinsic resistance, BCRP could not be detected. Moreover, also in samples from patients with drug-treated breast cancer or AML, no BCRP was detected. Although these data may point to a limited clinical relevance of BCRP in drug resistance, it should be taken into account that the breast cancer patients had shown a pronounced decrease in tumor mass after chemotherapy. Furthermore, in the AML patients, recurrence was independent of the drug types used, and, therefore, the results in the tumor cells tested thus far may not reflect true drug resistance. Because no *BCRP* mRNA data in human tumors have been published until now, we cannot compare the present results to data reported earlier. *BCRP* mRNA data should, however, be considered with caution in light of the presently observed BCRP staining in endothelial cells and desmoplastic stroma cells in the tumor samples.

In conclusion, our results indicate that BCRP is highly expressed in MX- and TPT-selected cell lines but not, or only at very low levels, in human tumors. Nevertheless, to reveal the potential contribution of BCRP to MDR, more extended studies are required. The BXP-34 Mab may be a valuable tool in these studies. Nevertheless, other BCRP-specific Mabs will be required that will allow BCRP detection with techniques suited for large-scale screening of tumor samples. We are currently making efforts to obtain such Mabs. Furthermore, studies are ongoing to examine the detailed normal tissue distribution of the BCRP protein. These studies may give clues regarding tumors that arise from BCRP-positive tissues and that are perhaps more likely to use the BCRP protein as a drug resistance mechanism.

## References

1. Moscow, J. A., Schneider, E., Ivy, S. P., and Cowan, K. H. Multidrug resistance. Cancer Chemother. Biol. Response Modif. Annu., *17*: 139–177, 1997.
2. Gottesman, M. M., Pastan, I., and Ambudkar, S. V. P-glycoprotein and multidrug resistance. Curr. Opin. Genet. Dev., *6*: 610–617, 1996.
3. Cole, S. P., and Deeley, R. G. Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. BioEssays, *20*: 931–940, 1998.
4. Paulusma, C. C., Bosma, P. J., Zaman, G. J., Bakker, C. T., Otter, M., Scheffer, G. L., Scheper, R. J., Borst, P., and Oude Elferink, R. P. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science (Washington DC), *271*: 1126–1128, 1996.
5. Kool, M., van der Linden, M., de Haas, M., Scheffer, G. L., de Vree, J. M. L., Smith, A. J., Jansen, G., Peters, G. J., Ponne, N., Scheper, R. J., Oude Elferink, R. P. J., Baas, F., and Borst, P. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc. Natl. Acad. Sci. USA, *96*: 6914–6919, 1999.
6. Doyle, L. A., Yang, W. D., Abruzzo, L. V., Krogmann, T., Gao, Y. M., Rishi, A. K., and Ross, D. D. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc. Natl. Acad. Sci. USA, *95*: 15665–15670, 1998.
7. Schneider, E., and Hunke, S. ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. FEMS Microbiol. Rev., *22*: 1–20, 1998.
8. Ross, D. D., Yang, W. D., Abruzzo, L. V., Dalton, W. S., Schneider, E., Lage, H., Dietel, M., Greenberger, L., Cole, S. P. C., and Doyle, L. A. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. J. Natl. Cancer Inst., *91*: 429–433, 1999.
9. Maliepaard, M., van Gastelen, M. A., de Jong, L. A., Pluim, D., van Waardenburg, R. C. A. M., Ruevekamp-Helmers, M. C., Floot, B. J., and Schellens, J. H. M. Overexpression of the *BCRP/MXR/ABCP* gene in a topotecan-selected ovarian tumor cell line. Cancer Res., *59*: 4559–4563, 1999.
10. Taylor, C. W., Dalton, W. S., Parrish, P. R., Gleason, M. C., Bellamy, WT, Thompson, F. H., Roe, D. J., and Trent, J. M. Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF7 human breast cancer cell line. Br. J. Cancer, *63*: 923–929, 1991.
11. Hazlehurst, L. A., Foley, N. E., Gleason-Guzman, M. C., Hacker, M. P., Cress, A. E., Greenberger, L. W., de Jong, M. C., and Dalton, W. S. Multiple mechanisms confer drug resistance to mitoxantrone in the human 8226 myeloma cell line. Cancer Res., *59*: 1021–1028, 1999.
12. Rogan, A. M., Hamilton, T. C., Young, R. C., Klecker, R. W. J., Ozols, and RF Reversal of Adriamycin resistance by verapamil in human ovarian cancer. Science (Washington DC), *224*: 994–996, 1984.
13. Eijdems, E. W., de Haas, M., Timmerman, A. J., Van der Schans, G. P., Kamst, E., de Nooij, J., Astaldi Ricotti, G. C., Borst, P., and Baas, F. Reduced topoisomerase II activity in multidrug-resistant human non-small cell lung cancer cell lines. Br. J. Cancer, *71*: 40–47, 1995.
14. Withoff, S., De Vries, E. G. E., Keith, W. N., Nienhuis, E. F., van der Graaf, W. T. A., Uges, D. R. A., and Mulder, N. H. Differential expression of DNA topoisomerase II-$\alpha$ and -$\beta$ in P-gp and MRP-negative, VM26, mAMSA and mitoxantrone-resistant sublines of the human SCLC cell line GLC(4). Br. J. Cancer, *74*: 1869–1876, 1996.
15. Marsh, W., and Center, M. S. Adriamycin resistance in HL60 cells and accompanying modification of a surface membrane protein contained in drug-sensitive cells. Cancer Res., *47*: 5080–5086, 1987.
16. Allen, J. D., Brinkhuis, R. F., Wijnholds, J., and Schinkel, A. H. The mouse *Bcrp1/Mxr/Abcp* gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. Cancer Res., *59*: 4237–4241, 1999.
17. Gluzman, Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell, *23*: 175–182, 1981.
18. Aruffo, A., and Seed, B. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc. Natl. Acad. Sci. USA, *84*: 8573–8577, 1987.
19. Evers, R., Kool, M., van Deemter, L., Janssen, H., Calafat, J., Oomen, L. C., Paulusma, C. C., Oude Elferink, R. P., Baas, F., Schinkel, A. H., and Borst, P. Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. J. Clin. Investig., *101*: 1310–1319, 1998.
20. Cui, Y. H., Konig, J., Buchholz, U., Spring, H., Leier, I., and Keppler, D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol. Pharmacol., *55*: 929–937, 1999.